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Donor-derived peripheral mononuclear cell DNA is associated with stable kidney allograft function

A randomized controlled trial

Ghasem Solgi,[1] Joannis Mytilineos,[2] Vijayakrishna Gadi,[3] Biswajit Paul,[3] Gholamreza Pourmand,[4] Abdolrasoul Mehrsai,[4] Behrouz Nikbin[5] and Ali Akbar Amirzargar[5,*]

[1]Immunology Department; School of Medicine; Hamadan University of Medical Sciences; Hamadan, Iran; [2]Transplantation Immunology; Institute for Clinical Transfusion Medicine and Immunogenetics Ulm; Department of Transfusion Medicine; University Hospital Ulm; Ulm, Germany; [3]Clinical Research Division; Fred Hutchinson Cancer Research Center; Seattle, WA USA; [4]Urology Research Center; Sina Hospital; [5]Molecular Immunology Research Center; Tehran University of Medical Sciences; Tehran, Iran

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A large body of literature has documented an inconsistent relationship of peripheral donor cell chimerism with alloimmune tolerance following kidney transplantation. We revisit this association with assays capable of quantifying cellular microchimerism with 150–1500-fold greater sensitivity than previously utilized allo-antibody based flow cytometric approaches. Forty renal transplant patients, 20 with concurrent donor bone marrow infusion (DBMI) and 20 control participants without infusion were prospectively monitored for peripheral blood microchimerism using donor polymorphism-specific quantitative real-time PCR. Thirty-eight patients were evaluated for microchimerism, 19 in each group. The frequency of testing positive for (95% vs. 58%, p = 0.02) and mean concentrations of microchimerism (115 ± 66 vs. 13 ± 3 donor genomes/million recipient genomes, p = 0.007), respectively, were higher in infused patients compared with controls. Thirty-one patients maintained stable graft function; 17 in the DBMI group vs. 14 in controls. Patients with stable graft function in the DBMI group compared with control patients harbored microchimerism more frequently (94% vs. 50%, p = 0.01) and at higher concentrations (123 ± 67 vs. 11 ± 4, p = 0.007), respectively. Significant correlation between dose of infused cells and microchimerism levels was found post-transplant (p = 0.01). Using very sensitive assays, our findings demonstrate associations between the presence and quantity of microchimerism with stable graft function in infused patients.

Introduction

Allograft acceptance occurs when a two-way immune response results in reciprocal clonal exhaustion-deletion, which is understood to be the seminal mechanism for acquired tolerance after transplantation. Microchimerism, the persistence of a small quantity of donor cells in the host, may be a prerequisite for the maintenance of this situation (induced clonal deletion) and this form of tolerance has been shown to depend on a balance between microchimerism and anti-donor immunity.[1-3] Based on the observation of persistent systemic microchimerism in long-term allograft recipients, a number of trials were initiated to test the hypothesis that donor bone marrow cell infusion (DBMI) administered concurrently with transplant could augment tolerance.[4-7] Miller et al.[8] reported significantly decreased chronic rejection and higher graft survival rates in the presence of chimerism in kidney recipients with DBMI vs. non-infused recipients during six years follow up. Additionally, chimeric cells derived from iliac crest of infused kidney recipients had an inhibitory effect on anti-donor response in mixed lymphocyte reaction (MLR) suggesting the presence of regulatory elements.[9] Similarly, in another study this inhibitory effect of chimeric cells in donor-specific MLR was shown for living, related donor kidney recipients with DBMI vs. non-infused patients.[10]

Although elegant preclinical studies strongly suggest the importance of donor cell chimerism for active maintenance of T-cell unresponsiveness, the role of such cells in human studies remains unclear.[11] In part, the inconsistent observations may be accounted for by the insensitive methodology—HLA-subtype specific flow cytometry to detect chimeric cells. With the advent of quantitative molecular techniques, microchimeric cells are detectable with up to 2–3 orders of magnitude greater sensitivity. Utilizing polymorphism-specific quantitative PCR, we therefore set out to determine whether the persistence of microchimerism following low-dose DBMI without intensified conditioning would be associated with stable allograft function.

Results

Clinical outcomes. Concurrent DBMI was well-tolerated and no graft vs. host disease was observed. Data given in **Table 1** summarize the demographics and clinical characteristics with

*Correspondence to: Ali Akbar Amirzargar; Email: amirzara@sina.tums.ac.ir
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Table 1. Demographics and transplantation characteristics

	DBMI (n = 20)	Controls (n = 20)
Recipient's age in years (Mean ± SD)	43 ± 14	45 ± 18
Recipient's gender (M/F)	(13/7)	(14/6)
Donor's age in years (Mean ± SD)	36 ± 13	32 ± 8
Pre-transplant PRA (by CDC)		
0–5%	18	19
5–10%	2	1
Number of HLA mismatches (A/B/DR)		
2/6	1 (5%)	0 (0%)
3/6	5 (25%)	3 (15%)
4/6	6 (30%)	5 (25%)
5/6	4 (20%)	7 (35%)
6/6	4 (20%)	5 (25%)
Median	4	5
Etiology of ESRD		
Chronic Glomerulonephritis	2	0
Diabetic nephropathy	4	7
Polycystic kidney disease	1	3
Hypertension	8	2
Renal stone	1	3
Unknown	4	5
Cold ischemia time minutes (Mean ± SD)	52 ± 9.6	51 ± 14.9
Warm ischemia time minutes (Mean ± SD)	4.2 ± 0.8	4.1 ± 0.85
CMV infection post-transplant	7	7
DGF	3	4
Maintenance immunosuppressive regimens		
Cyclosporine A (mg/day) (mean ± SD)	119.2 ± 25.3	140.4 ± 37.5[a]
Mycophenolate Mofetil (gr/day) (mean ± SD)	2.46 ± 0.51	2.50 ± 0.65
Methyl prednisolone (mg/day) (mean ± SD)	5.44 ± 1.93	6.25 ± 2.26

None of the variables were statistically significant between both groups except for cyclosporine A dosage ([a]p = 0.04).

no statistically significant differences between both groups of patients except for cyclosporine A dosage at the end of the follow-up period. The number of HLA mismatches (A/B/DR) was nearly the same between both groups and all patients received an allograft with 2–6 HLA mismatches.

Comparison of patient and graft survival between two groups during the 28 mo of mean follow up showed no significant differences. Overall patient survival was 100% and 95%, p = 1.0 and graft survival was 95% and 80%, p = 0.34, respectively, between the DBMI and control groups. Of the 40 patients, 9 (22.5%) showed acute rejection episodes (ARE), 3 in the DBMI group and 6 in the control group (p = 0.45). All 3 patients with ARE in the DBMI group were noncompliant with immunosuppressive drugs and required anti-thymocyte globulin for the management of rejection. Among the 6 patients with ARE in the control group, two cases were acute humoral rejections, with one of them eventually losing his graft. One more case showed acute humoral and cellular rejection at day 25 and returned to dialysis (graft loss). The remaining three controls with clinical ARE responded to immunosuppressive treatment. Overall, four patients lost their grafts entirely within the first year after transplantation; one from the DBMI group who underwent transplant nephrectomy because of uncontrolled bleeding 28 d after surgery, and 3 cases in the control group [above described acute rejections (2 cases) and one death with functioning graft] (p = 0.60).

Delayed graft function was observed in 7 patients (4 in control vs. 3 in DBMI, p = 1.0, **Table 1**). Although, there appeared to be a trend toward need for lower dose of methyl prednisone, cyclosporine A and MMF in infused patients than in controls at the end of second year, this difference was significant only for cyclosporine A (p = 0.04, **Table 1**).

The mean serum creatinine levels in the first year post operatively were marginally lower in infused patients vs. controls (1.60 ± 0.37 vs. 1.91 ± 0.57, p = 0.08). Moreover, serum creatinine levels at the time of rejection episodes were lower but insignificantly different in infused patients vs. controls (3.35 ± 0.90 vs. 5.8 ± 3.56, p = 0.15). DBMI was not associated with more incidence of CMV infection over the follow-up period (**Table 1**). During the follow-up period, only one case from the control group developed biopsy-proven chronic rejection at the end of the second year after transplantation.

Susceptibility to post-transplant morbidity (requiring hospitalization) including viral, fungal and bacterial infection, and rising creatinine levels were not significantly different between the two patient groups (45% in DBMI and 40% in controls). Hospitalization due to CMV infection was the same for both groups (4 cases).

Microchimerism analysis. Quantification of peripheral blood mononuclear cell microchimerism in serial samples during first year post transplantation (days 7, 14, 30, 90, 180 and 360) as well as in pre-transplant samples was performed using real-time polymorphism-specific quantitative PCR. Overall, 38 cases were available for microchimerism analysis (19 patients in DBMI and 19 in the control group). For ease of expression, concentrations of microchimerism are reported as genome equivalent of donor cells per million of recipient cells (gEq/10^6 cells). The median DNA equivalent total number of cells tested was similar between groups, 1.16 x 10^5 gEq in controls vs. 1.12 x 10^5 gEq in infused patients.

The mean concentration of post transplant microchimerism in DBMI patients was higher at all time intervals compared with controls, with significant differences observed at days 7 (p = 0.001) and 14 (p = 0.05) (**Table 2**). Additionally, the

number of patients testing positive for microchimerism during the first week in the DBMI group was approximately double that observed in controls (84% vs. 44%, p = 0.02, **Table 2**). None of the patients tested positive for microchimerism at all time points.

Table 3 summarizes microchimerism results for the entire first post-transplant year. Frequency of patients testing positive in the first year post-transplant and mean concentrations of microchimerism were significantly higher in the DBMI group compared with controls (95% vs. 58%, p = 0.02; and 115.3 ± 65.5 vs. 12.7 ± 3.4 gEq/mil, p = 0.007, respectively, **Table 3**). When analysis was restricted to patients with stable graft function, the DBMI group harbored microchimerism more frequently and at higher concentrations than the control group (94% vs. 50%, p = 0.01; and 123 ± 67.4 vs. 10.6 ± 3.8 gEq/mil, p = 0.007, respectively). Significant differences for microchimerism concentrations in DBMI and control patients with normal graft function were found at days 7 and 30 post-transplant (**Fig. 1**). Significant differences in microchimerism in recipients with ARE between groups was not observed but the number of events available for analysis was small. Pre-transplant testing for microchimerism showed that only two cases were positive for peripheral microchimerism; one in the DBMI group and the other in the controls who had 7 gEq/mil and 20 gEq/mil of recipients' cells.

In the DBMI group, cell dose was correlated with microchimerism concentrations at day 7 (p = 0.01), day 14 (p = 0.03), and day 90 (p = 0.02) (**Fig. 2A–C**). Moreover, there was a significant inverse correlation between the microchimerism concentrations in the first week and serum creatinine levels at months 1, 6 and 12 (**Fig. 2D–F**), and also between microchimerism concentrations at month 1 and serum creatinine at days 14 and 30 post transplantation (**Fig. 2G and H**). Finally, an inverse correlation was found between dose of infused cells and serum creatinine levels at month 1 (r = -0.412, p = 0.07).

Post-transplant anti-HLA antibodies and presence of microchimerism. The results of anti-HLA antibody screening and identification for both groups have been described previously by Solgi et al.[12] Donor-specific antibodies (DSA) were not detected in microchimerism-positive patients among the infused group regardless their ARE status. In total, 5 patients showed both DSA and non-DSA; one in the DBMI group (without ARE) and 4 in the controls (3 with ARE). Of these five patients only 2 cases with ARE (in controls) were positive for microchimerism. In addition, 5 more cases harbored non-DSA only, all of them being positive for microchimerism: 4 in the DBMI group (2 with ARE and 2 without ARE) and one in the controls (with rejection).

The mean percentage of post-transplant panel reactive antibodies (PRA) was 16% in DBMI patients (4 cases) and 36% in the controls (3 cases). PRA positive cases did not show significant differences with respect to microchimerism concentrations (35.7 ± 29.9 gEq/10^6 in infused group vs. 32.7 ± 17.2 gEq/10^6 in the controls, p = 0.82).

Table 2. Frequency and concentrations (gEq/10^6 host cells) of peripheral microchimerism

	Control (n = 19)	DBMI (n = 19)	p values*
Day 7	8/18 (44%) 21.5 ± 8.8	16/19 (84%) 374 ± 128	0.02 0.001
Day 14	6/17 (35%) 16.7 ± 8.1	12/19 (63%) 87 ± 40	0.18 0.056
Day 30	5/17 (30%) 11.7 ± 6.2	9/19 (47%) 36 ± 15	0.44 0.20
Day 90	8/17 (47%) 12.9 ± 5.07	6/15 (40%) 45 ± 18	0.96 0.51
Day 180	2/13 (15%) 0.77 ± 0.62	2/10 (20%) 34.9 ± 34.2	0.79 0.70
Day 360	0/9 (0.0%)	0/5 (0.0%)	-

*Two-tailed p values by Fisher exact test (for frequency) and Mann-Whitney U test (for concentrations, mean donor gEq/10^6 host cells ± SEM).

Table 3. Summary of microchimerism in all patients and stratified by graft function

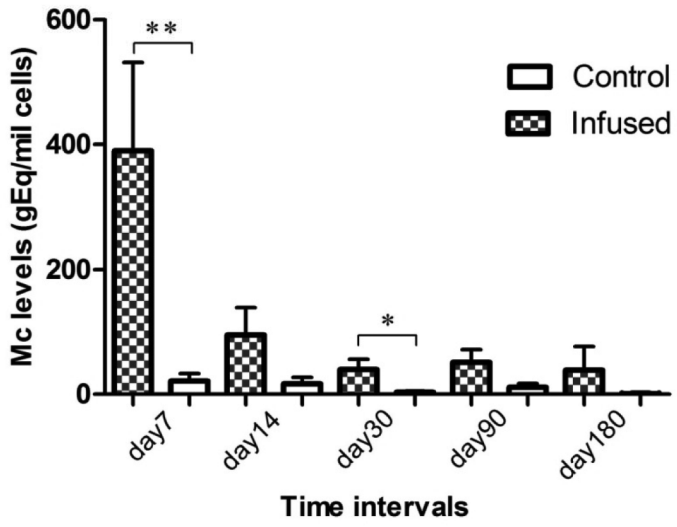
	Microchimerism	Donor gEq/10^6 cells*
All patients		
Control (n = 19)	11/19 (58%)	12.7 ± 3.4
DBMI (n = 19)	18/19 (95%)	115.3 ± 65.5
	p = 0.02	p = 0.007
SGF		
Control (n = 14)	7/14 (50%)	10.6 ± 3.8
DBMI (n = 17)	16/17 (94%)	123 ± 67.4
	p = 0.01	p = 0.007
ARE**		
Control (n = 5)	4/5 (80%)	20.6 ± 7.3
DBMI (n = 2)	2/2 (100%)	43.7± 37.7
	p = ns	p = ns

**One case from each group was excluded from Mc analysis; in the DBMI group, because of uncontrolled bleeding treated with multiple blood transfusions; and in the control group, because of DNA contamination in post-transplant specimen. *Mean ± SE; ns, not significant.

Discussion

In prior studies of DBMI at the time of organ transplantation, a correlation with better allograft survival was observed, and in some cases, weaning of immunosuppressive treatment was possible.[10,13,14] These proof-of-principle results subsequently spurred interest in simultaneous non-myeloablative hematopoietic cell and kidney transplantation approaches.[14-17] Monaco et al. used DBMI in kidney allograft recipients concomitant with anti-lymphocyte globulin-induction therapy. Subsequently, several clinical trials based on Monaco's model have been conducted to date, not only in kidney but also in liver, heart, lung and pancreas transplantation.[15,17,19,20]

In our pilot study, living unrelated DBMI was provided to kidney allograft recipients from the same donor immediately post procedure in order to augment peripheral microchimerism. We evaluated the association of microchimerism on early allograft function (SGF vs. acute rejection) and conventional alloimmune response such as anti-HLA antibodies and inflammatory markers. It is noteworthy that the current study is small and therefore



Time intervals	Mc levels (gEq/10^6 cells) DBMI (n=17)	Control (n=14)	p values*
Day 7	389.6 ± 142.3	21.0 ± 12.9	**0.002**
Day 14	95.3 ± 43.9	16.7±10.4	0.09
Day 30	39.8 ± 16.6	3.2 ± 1.8	**0.03**
Day 90	51.5 ± 20.3	11.4 ± 5.7	0.26
Day 180	38.78 ± 38.03	0.83 ± 0.67	0.62

Figure 1. Microchimerism levels (gEq/10^6 host cells) in different time intervals for patients with SGF from both groups. A significant difference was identified at days 7 and 30 post-operatively. *Mann-Whitney U test, 2-tailed p values.

was not powered to examine graft survival or overall patient survival.

Using a highly specific and sensitive panel of polymorphism specific quantitative PCR to target donor sequences in microchimeric cells, we determined that the frequency of patients testing positive for, and mean concentrations of microchimerism were significantly higher in the DBMI group compared with controls during first year following the procedure. Stable graft function was also correlated with both presence and concentrations of microchimerism, despite the small numbers of patients evaluated in the trial. This association was present in the very early weeks post-transplantation and was durable for the course of the first year of observations taken.

Albeit at lower levels than the DBMI group, peripheral microchimerism in our control patients was also detected frequently. This is different from findings reported by Morales et al.[21] who did not identify microchimerism in non-infused allograft recipients. In contrast to De Pauw et al.[22] we observed a significantly higher frequency of patients with peripheral microchimerism in the DBMI group vs. the control group, in spite of almost similar quantity of donor $CD34^+$ cells infused and the omission of OKT3 induction therapy in our patients. The most likely explanation for these differences is methodological because of the substantially higher sensitivity of our detection method. However, the presence of microchimerism before transplant in two female recipients who had former female fetuses indicate that these cases may have unknown to us shared HLA sequences with the unrelated donor. As we couldn't test the maternal or fetal microchimerism (due to unavailable samples from their family members), one possible explanation for the presence of low-level peripheral microchimerism in some patients could be this kind of pre-existing chimerism.

With regards to alloimmune response markers, we observed decreased serum concentrations of IFNγ and sCD30, lower percentage of post transplant PRA and lower strength (titer) of anti-HLA antibodies in infused patients compared with controls (described previously by this center).[12,23] Meanwhile, donor-specific antibodies were not detected in the presence of microchimerism in DBMI group, possibly indicating enhancement of tolerance. The results of clinical-immune surrogate markers in our patients are consistent with unresponsiveness in similar studies,[9,10,24,25] with the limitation that patient-derived resources did not permit direct demonstration of immunologic unresponsiveness against donor cells (MLR test or similar assays). It is

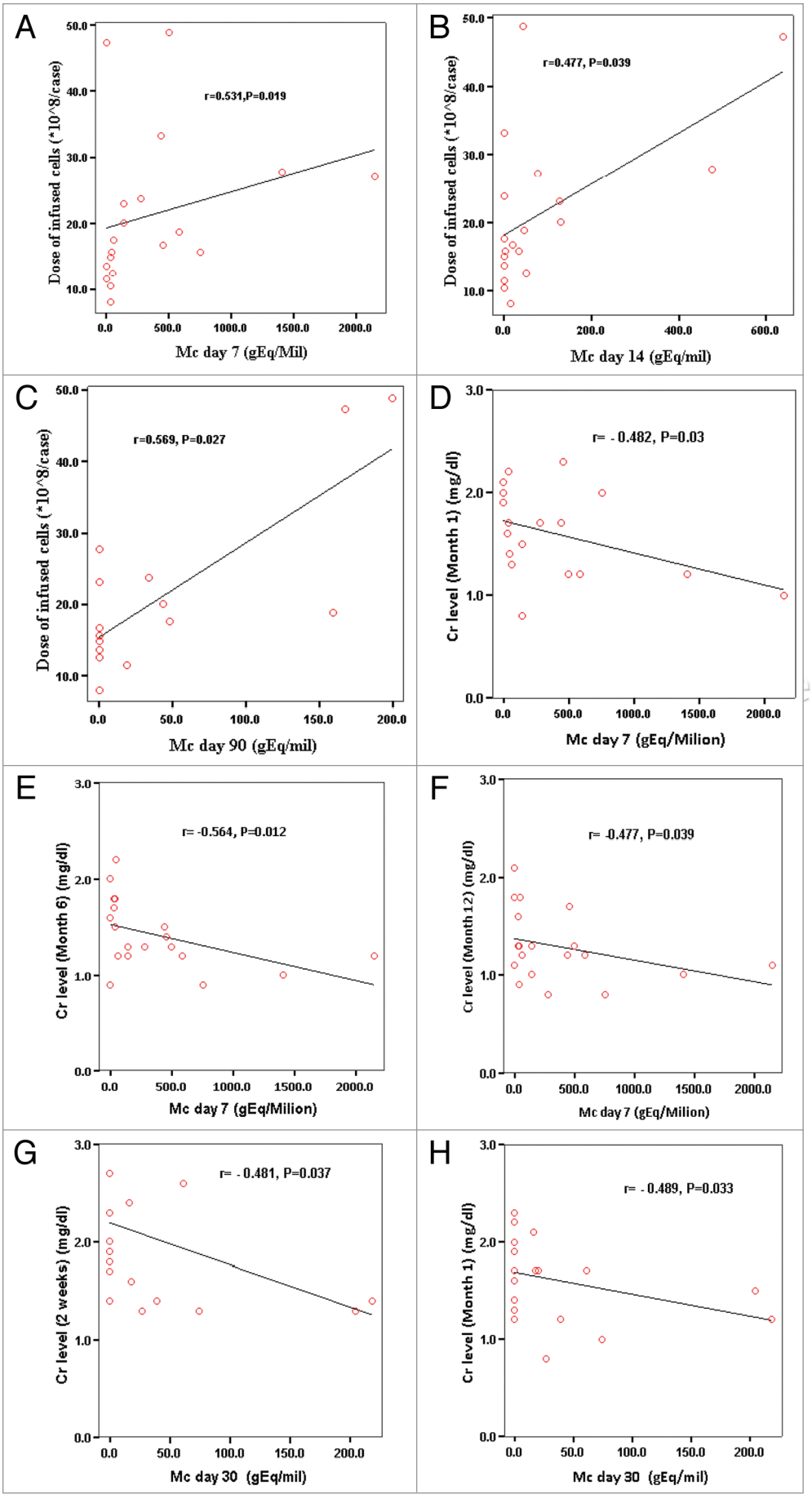


Figure 2. Bivariate correlation analysis for microchimerism levels, cell dose and serum creatinine levels among infused patients. (A–C) Direct correlation between dose of infused cells (*10^8/recipients) and microchimerism concentrations (gEq/10^6 host cells) at day 7, 14 and 90. (D–F) Inverse correlation between microchimerism concentrations at day 7 and serum creatinine concentrations at month 1, 6 and 12. (G and H) Inverse correlation between microchimerism concentrations at day 30 and serum creatinine concentrations at day 7 and month 1 post transplantation.

noteworthy that there was also a trend toward decreased drug-mediated immunosuppressant (cyclosporine) in infused patients by the end of second year post transplant.

The use of DBMI in our protocol appears safe. Ciancio et al.[25] who utilized antibody-based induction regimens with maintenance therapy reported that six-year follow up of 63 infused patients and 213 controls had a higher prevalence of CMV infection in infused patients and a similar incidence of ARE in both groups. Instead, we observed more ARE in the controls (30% vs. 15%) and a similar rate of CMV infection for both groups. Notably, patients who suffered ARE in either study harbored decreased microchimerism concentrations, suggestive for an early role of DBMI on allograft function.

In conclusion, our highly specific and sensitive assays for microchimerism detection may clarify the inconsistent association of donor-derived cellular microchimerism in renal transplantation outcomes. As numerous techniques ranging from cellular to DNA-based methods are utilized for detection of chimerism, nevertheless, to avoid misinterpretation of the relation between microchimerism and outcome of allograft such as rejection, a more sensitive and precise and standardized method must be implemented in this kind of studies.[26,27]

In this study, DBMI was well tolerated without any observed adverse effects. Remarkably, in the absence of HLA matching, antibody-induction regimen and additional maintenance therapy, chimerism augmentation was observed in almost all patients of the DBMI group. Also, there was a statistically significant association between the presence and concentrations of peripheral chimerism with improved graft function in infused patients. Conversely, the poorer outcomes including the higher number of ARE, more graft loss and low-grade continuous deterioration of renal function in the controls were associated with lower frequencies and concentrations of peripheral chimerism. Our data suggest a trend toward an early effect of the DBMI on allograft outcomes and alloimmune response. The absence of persistently detectable microchimerism following DBMI might be because of the administration of an insufficient number of cells. Because of substantial differences in several important variables including immunosuppressive drugs regimen, dose of infused cells, type of the cells administered, time of infusion, HLA matching and method for detection of microchimerism, comparison of this study to other published approaches should be interpreted cautiously. Ultimately, a longer follow-up of patients in our study as well as more thorough immunological assessment of immune responses in subsequent studies of DBMI in kidney transplantation are necessary in order to assess the impact on long-term allograft function and survival. More importantly to find the exact role of donor-derived cells, it would be of interest to specifically identify cells derived from the bone marrow graft but that was not possible with specimens available for the current study.

Finally, our data offer additional support for Starzl's hypothesis regarding the role of chimerism in allograft outcomes.[2,28] Based on this hypothesis, augmentation of chimerism results in increased reciprocal immune interactions between donor and recipient which in the presence of conventional immunosuppressive drugs, may lead to host-vs.-graft immunity becoming more susceptible for clonal exhaustion and deletion rather than the donor bone marrow cell immune response against the recipient (graft vs. host).[1,29] Because of lower susceptibility of infused donor marrow cells to immunosuppressive therapy,[30] a functional immune equilibrium postulated by Starzl et al.[4] may be established even with low dose infusion of donor cells. Remarkably, absence of clinical graft-vs.-host disease, fewer ARE (and more importantly the reversible status of those rejection episodes), and no documented chronic rejection in our DBMI patients are all possible manifestations of Starzl's functional equilibrium.

Patients and Methods

Study design. This pilot study was a single center randomized controlled clinical trial designed to determine whether the persistence of microchimerism following low-dose DBMI in recipients of kidney allograft would be associated with stable graft function.

Ethics statement. The Ethics Committee of Tehran University of Medical Sciences has approved the project in accordance with the tenets of the Helsinki declaration and the national ethical guideline for medical research.

Participants and transplantation protocol. Between March 2005 and July 2007, 40 living unrelated donor primary kidney recipients who consecutively enrolled in our study were included and prospectively clinically followed for a mean period of 28 mo (24–33 mo). Enrollment and participation flow through the study is shown in **Figure 3**. Written informed consent was obtained from all donors and recipients according to protocols approved by the TUMS research ethics committee. The exclusion criteria were previous transfusion, re-transplantation and former pregnancy with male fetus, genetic diseases and morbid obesity. Consenting patients were divided in a randomized fashion into two groups consisting of 20 subjects receiving donor bone marrow cells infusion concurrent with their kidney allograft (DBMI group, informed consent was obtained for infusion and follow up) and 20 controls receiving a kidney allograft only (informed consent was obtained for follow up). Both groups were given the same baseline conventional immunosuppressant regimen that was instituted 24 h prior to renal transplantation. The protocol consisted of the triple drug regimen: cyclosporine A (6 mg/kg/day BD), mycophenolate mofetil (MMF) (2 gr/day BD) and prednisolone (2 mg/kg/day). The patients were not preconditioned with any cytoablative or cytoreductive regimen. No immunomodulatory monoclonal antibodies or ATG were given.

Genotyping was performed by standard PCR-SSP technique for all donors and recipients to determine the HLA-A, B and DRB1 alleles (HLA-A B DR low resolution typing kit).

Bone marrow cells preparation and infusion (intervention protocol). Donor bone marrow cells were obtained from iliac crest by aspiration of 150–200 mL of bone marrow specimen at the time of donor nephrectomy. Afterwards, mononuclear cells of those samples were isolated using hydroxyethyl starch (HES 6%, plasmasterile, Fresenius) as described by Adkins D et al. with a brief modification. One-half ml aliquots of MNCs suspension in HES were analyzed for absolute count of total nucleated cells,

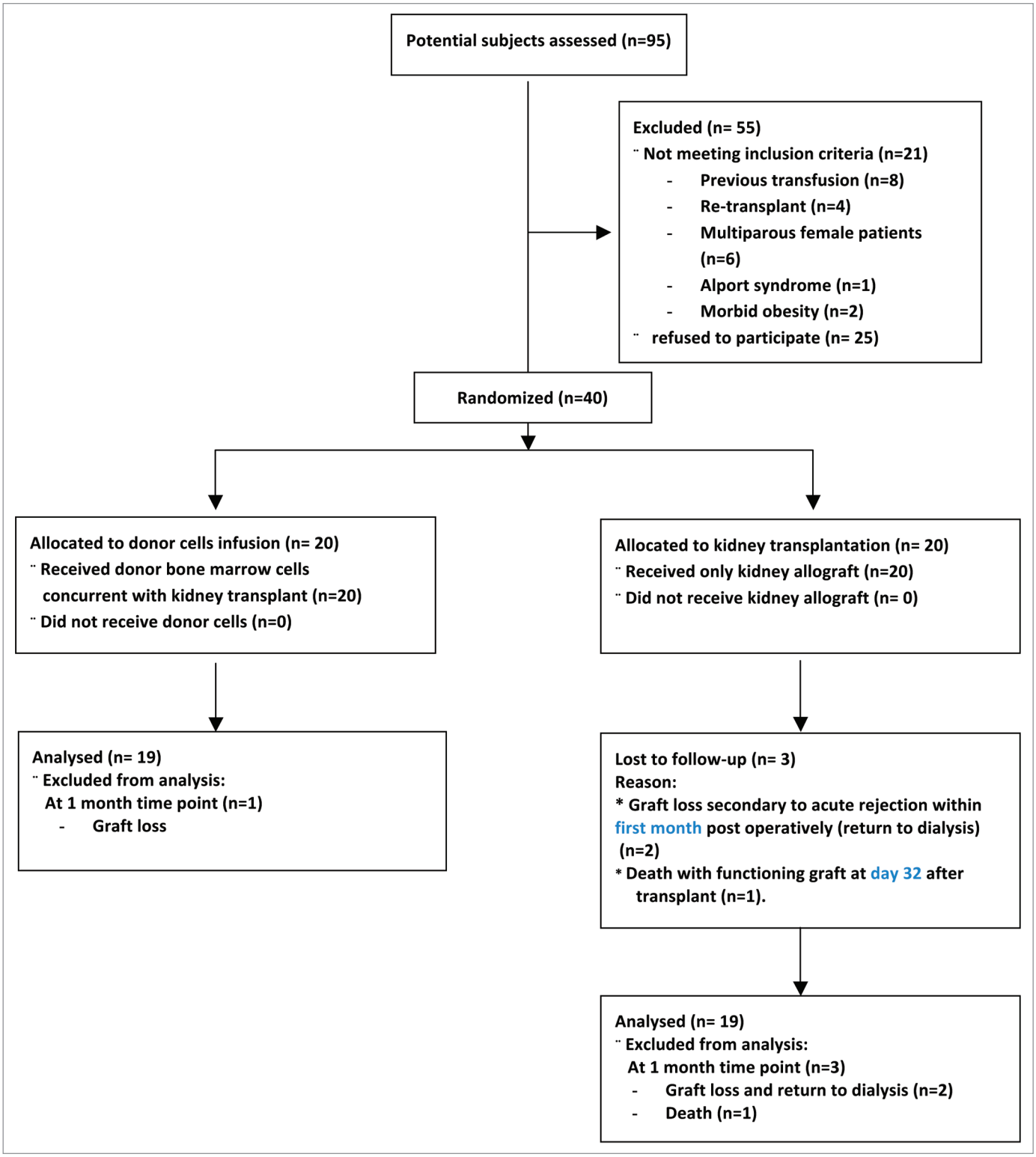


Figure 3. Flow diagram of the study.

and flowcytometric determination of percentage and absolute number of $CD34^+$ $CD45^+$ hematopoietic progenitor cells (by RPE conjugated anti-CD34 and FITC labeled anti-CD45 and isotype matched negative control, DACO, Denmark) according to the ISHAGE guidelines.[32] The average number of donor cells which was infused immediately post-operatively was $2.19 \times 10^9 \pm 1.13 \times 10^9$ mononuclear cells/recipient. The average number of $CD34^+$ progenitor cells was $2.66 \times 10^7 \pm 1.70 \times 10^7$.

Clinical follow up. Primary outcome measures were the frequencies of acute rejections or rejection episodes, delayed graft function (DGF), chronic rejection, presence and quantity of peripheral microchimerism and stable graft function for each group. Clinical acute rejection was considered after an increase of creatinine by 0.3 mg/dl or greater from the baseline and confirmed by renal biopsy. Delayed graft function (DGF) was defined as a requirement for dialysis within the first week postoperatively because of rising serum creatinine after ruling out of other causes of graft dysfunction. A graft was considered as lost upon return of the patient to dialysis, transplant nephrectomy or death. Patients with no history of clinical and/or biopsy proven rejection and with good functioning graft as judged by serum creatinine level (<1.5 mg/dl) were considered as stable graft function. Secondary end points were incidence of CMV or other infection and need for hospitalization due to any reason after discharge from hospital.

Microchimerism assays. Serial peripheral blood phlebotomy was performed pre-transplant and again on days 7, 14, 30, 90, 180 and 360 post-transplant from all 40 patients. Ficoll-purified peripheral blood mononuclear cells were processed to obtain total genomic DNA utilizing a commercially available kit (Qiagen). DNA was shipped and stored frozen until use for PCR assays.

Development of the quantitative PCR method to detect peripheral blood mononuclear cell microchimerism was previously described in reference 33. In brief, routine HLA genotyping was reviewed to identify polymorphisms unique to the donor with respect to the recipient. Whenever possible, donor HLA polymorphisms were targeted using a large panel of available HLA assays.[34] The mismatched HLA alleles which used for detection and quantification of microchimerism were HLA-DR1, DR2, DR4, DR7, DR8, DR10, DR14, DQB1*03, DQB1*0301 and DQA1*05.

If donor-recipient pairs did not have targetable HLA disparity, additional genotyping was performed so that non-HLA assays (*GST, AT3*) could be utilized as an alternative. One female recipient paired with a male donor had microchimerism identified with Y chromosome gene *DYS14*. All assays were developed to have equivalent sensitivity (generally 1 donor genome per 1.2 x 10^5 recipient genomes tested). Specificity for assays was confirmed by testing each combination of primers and probes against genomic DNA from a large panel of well-characterized HLA specific lymphoblastoid cell lines (Gift of International Histocompatibility Workshop Group). Concentrations of chimeric DNA were determined by plotting cycle of amplification detection against a calibration curve of 0, 0.5, 1, 5, 10, 50, 100 and 500 HLA or non-HLA genomic equivalents diluted in a background of 1–2 x 10^4 genomic equivalents of a non-similar HLA type, and then divided by the total number of genomes tested as determined by concurrent β globin (BGLOB) quantitative PCR. Results from 6–12 aliquots were summed to obtain total chimeric quantity. Two aliquots were tested for BGLOB, results averaged and multiplied by the number of aliquots tested for chimeric sequence. All assays were performed using TaqMan chemistry according to the manufacturer's directions (Applied Biosystems). Three hundred n*M* of each amplification primer and 100 nM of the dual-labeled probe were used. The amplification conditions consisted of an initial incubation at 50°C for 2 min, followed by incubation at 95°C for 10 min, 45 cycles of 95°C denaturation for 15 sec and extension at 56–64°C for 1 min.

Immunologic assays. Pre-transplant panel reactive antibodies (PRA) analysis and WBC cross match were done by the complement dependent cytotoxicity method. Screening and identification of anti-HLA antibodies prior and after transplantation were done for all patients by ELISA (Ab Screen, HLA class I and II, Ab Identification, HLA class I and II, Biotest) according to manufacturer's instructions.

Randomization. A priori randomization sequence was determined by random-numbers table, such that even numbers were allocated to receive donor bone marrow cells infusion concurrent with renal transplantation. The allocation sequence was then concealed from the staff involved in the enrolling and assessing participants in sequentially numbered sealed and stapled envelopes.

The patients themselves unsealed the envelopes at the time of randomization. Neither patients nor clinicians were blinded as to intervention but, outcome assessors, executer of Para-clinical tests and data analyzing were kept blinded to the allocation. This work had received approval by the TUMS Research Ethics Committee. The study was conducted according to the guidelines set out in the Declaration of Helsinki.

Statistical analysis. Statistical analysis was performed using SPSS, version 11.5 for Windows. Data were represented as mean ± SD or mean ± SE. Groups were compared using the Chi-square and Fisher's exact tests for categorical variables. The Student t-test for normally distributed data and Mann-Whitney U test for not normally distributed variables was used. Kaplan-Meier estimates and log-rank statistics were used for comparison of patients and graft survivals between two groups. Also, correlation between the concentrations of microchimerism, serum creatinine and dose of infused cells was calculated using Spearman rank correlation test.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Authors' Contribution

G.S. designed and performed experiments and wrote the manuscript, J.M. participated in performance of the research and in the writing of manuscript, V.K.G. participated in performance of the research, data analysis and in the writing of manuscript, B.P. performed experiments, G.P. participated in research design and performance, A.M. participated in research design and performance, B.N. participated in research design and in the writing

of the manuscript, A.A. designed and supervised the experiment and wrote the manuscript. All co-authors have read and approved the final manuscript.

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